Selection and Characterization of a Carrot Cell Line Tolerant to Glyphosate[1]

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ABSTRACT

Cultured carrot (*Daucus carota* L.) cells were adapted to growing in 25 millimolar glyphosate by transfer into progressively higher concentrations of the herbicide. Tolerance was increased 52-fold, and the adaptation was stable in the absence of glyphosate. The uptake of glyphosate was similar for adapted and nonadapted cells. Activity of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase was 12-fold higher in the adapted line compared to nonadapted cells, while activities of shikimate dehydrogenase and anthranilate synthase were similar in the two cell types. The adapted cells had higher levels of free amino acids—especially threonine, methionine, tyrosine, phenylalanine, tryptophan, histidine, and arginine—than did nonadapted cells. Glyphosate treatment caused decreases of 50 to 65% in the levels of serine, glycine, methionine, tyrosine, phenylalanine, and tryptophan in nonadapted cells, but caused little change in free amino acid levels in adapted cells.

The adaptation reported here supports the growing body of evidence linking tolerance to glyphosate with increased levels of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase. The elevated levels of aromatic amino acids, which may confer resistance in adapted cells, suggest that control of the shikimate pathway may be altered in these cells.

The overproduction of enzymes required for growth has been observed in inhibitor-resistant lines of both mammalian (1, 10) and plant (3, 19) cells. In the case of the mammalian cells, the increases were due to gene amplification. The shikimic acid pathway enyme EPSP[2] synthase has been found to be strongly inhibited by the broad-spectrum herbicide glyphosate (*N*-[phosphonomethyl]glycine) (15), and adaptation of plant cells to this herbicide has been accompanied by large increases in the extractable activity of this enzyme (3).

While higher levels of EPSP synthase activity have been reported for cells growing in glyphosate, the effect of this change on levels of aromatic amino acids has not been reported. In this study, a carrot cell line with stable tolerance to glyphosate was characterized with respect to herbicide uptake, activities of some shikimate pathway enzymes including EPSP synthase, and free amino acid levels.

[2] Abbreviations: EPSP synthase, 5-enolpyruvylshikimic acid-3-phosphate synthase (EC 2.5.1.19; 3-phosphoshikimate 1-carboxyvinyltransferase); SP, shikimate-3-phosphate; PEP, phosphoenolpyruvate.

MATERIALS AND METHODS

Adaptation of Cells. Garden carrot (*Daucus carota* L. cv Danvers) cells were cultured in a defined medium as previously described (14). Cells that grew slowly in the presence of 0.25 mM glyphosate were transferred into medium containing progressively higher concentrations of the herbicide. Adapted cells were maintained for about 1 year (15 transfers, or about 60 cell generations) on 25 mM glyphosate. Cells were subsequently maintained on glyphosate-free medium for about 2 years (120 generations) prior to the experiments reported here.

Growth Study. Growth responses of adapted and nonadapted cells to glyphosate were determined by transferring 0.5 g fresh weight of cells into 100 ml of medium in 250-ml Erlenmeyer flasks. Fresh weight increase was determined after 10 d of incubation on a reciprocating shaker (80 rpm) at 27°C. Three replicates were included.

Glyphosate Uptake. Two g of cells in log phase were transferred into 100 ml medium containing 910 cpm/ml of (methyl-^{14}C) glyphosate and a total glyphosate concentration of 0.25 mM. Three replicate flasks were used for each cell type. At 1, 2, 4, 8, 24, and 48 hr after transfer, 10-ml aliquots were taken and cells were collected on Miracloth. After washing three times with 5 ml of medium containing 0.25 mM unlabeled glyphosate, cells were weighed and dispersed in gel formed by adding 4 ml H$_2$O and 15 ml Aquasol. Radioactivity was determined with a Packard scintillation spectrometer.

Amino Acids. Five g of cells in early stationary phase (10 d after transfer) were inoculated into 100 ml fresh medium in 250-ml flasks. Four replicate flasks were included for each cell type. After 4 d of incubation, 20-ml aliquots were taken for free amino acid extraction and determination. Just after sampling, filter-sterilized glyphosate stock solution (100 mM) was added to two flasks of each cell type to give a glyphosate concentration of 0.25 mM. After an additional 48 h of incubation, samples were again taken for determination of amino acid content.

Cells were collected on Miracloth, and approximately 1 g of cells was extracted twice with 80% ethanol. The extracts were dried under vacuum, then washed three times with diethyl ether to remove lipids. The residue was taken up in Li citrate buffer (pH 2.2), and amino acids and ammonium were analyzed using a Beckman 119Cl amino acid analyzer.

Enzymes. EPSP synthase and phosphatase activities were assayed using extract from cells harvested 7 d after inoculation. Cells were collected by vacuum filtration, frozen with liquid nitrogen, and then added with stirring to four volumes (w/v) of 50 mM Hepes/KOH buffer (pH 7.0) containing 1% (w/v) Polyclar AT (GAF Corp.) and 5 mM mercaptoethanol. After 30 min at 0°C, the extract was passed through four layers of cheesecloth and centrifuged 30 min at 30,000g. The pellet was discarded and solid (NH$_4$)$_2$ SO$_4$ (561 mg/ml) was added to the supernatant with stirring. The precipitate was harvested by centrifugation (10 min

at 30,000g) and dissolved in a minimum volume of 50 mM Hepes/KOH buffer (pH 7.0). The extract was assayed immediately.

EPSP synthase activity was determined as the release of phosphate from SP and PEP using the malachite green method described by Lanzetta *et al.* (12), except that Tergitol NP10 was used instead of Sterox. Phosphatase activity, when measured using either SP or PEP as a substrate, was effectively inhibited by the addition of ammonium heptamolybdate to the assay mixture (16). The assay contained 50 mM Hepes/KOH (pH 7.0), 1 mM PEP, 1 mM SP, and 0.1 mM ammonium heptamolybdate in a total volume of 0.1 ml. After preincubating the assay mixture for 5 min at 30°C, the reaction was started by adding 10 μl of extract. The mixture was incubated at 30°C for 5 min and stopped by the addition of the phosphate reagent. As phosphatase activity was inhibited by only 98.6% by 0.1 mM ammonium heptamolybdate, the measured activities were corrected for the remaining phosphatase activity.

Phosphatase activity was determined by measuring the release of Pi from PEP. The assay procedure was as described above, except that SP and ammonium heptamolybdate were omitted from the assay mixture.

Anthranilate synthase and shikimate dehydrogenase activities were assayed using extract from cells harvested 6 d after inoculation. Using a glass-glass homogenizer, cells were dispersed in one volume (w/v) of buffer (200 mM Tris-HCL [pH 7.5], 0.2 mM EDTA, 10 mM MgCl₂, 10 mM glutamine, 60% glycerol) and 2 mg/g fresh weight of DTT and PVP. The homogenate was passed three times through a nitrogen pressure cell (35 kg/cm²) at 4 to 5°C. After centrifugation for 10 min at 27,000g, one volume of supernatant was combined with two volumes of room temperature saturated (NH₄)₂SO₄ in water, and centrifuged as before. The pellet was resuspended in one-half volume of extraction buffer for use in the assay.

Anthranilate synthase activity was determined by monitoring the appearance of anthranilate photofluorometrically (J. Brotherton, unpublished data), using 340 and 400 nm as excitation and measuring wavelength, respectively.

Shikimate dehydrogenase activity was determined as shikimate-dependent NADPH formation using the reaction mixture of Dennis and Balinsky (7). NADPH formation was monitored fluorometrically, with excitation and measuring wavelengths of 340 and 455 nm, respectively.

RESULTS

Adaptation and Growth in Glyphosate. The adaptation process for cultured carrot cells was similar to that described for cultured cells of *Corydalis sempervirens* (3). In the present study, the adaptation to glyphosate appears to be stable; the ability of the cells to grow in high concentratons (>10 mM) of glyphosate was recently reconfirmed (data not shown) after an additional year of growth in the absence of the herbicide, following the two years of glyphosate-free maintenance preceding the present study.

The concentrations of glyphosate required to inhibit growth by 50% were 0.12 and 6.3 mM for nonadapted and adapted cells, respectively (Fig. 1). This indicates a 52-fold increase in tolerance in adapted cells. the increases in fresh weight during 10 d of incubation in medium without glyphosate were 7.7 and 15.5 g, respectively, for adapted and nonadapted cells. This slow growth by adapted cells has been observed repeatedly in other studies (data not shown).

Glyphosate Uptake. The uptake of [¹⁴C]glyphosate from the medium by adapted and nonadapted cells is shown in Figure 2. As was previously reported (14), glyphosate uptake by these cells was rapid and linear during the first 8 h after transfer, then intracellular radioactivity declined during the next 40 h, apparently approaching an equilibrium level in the cells about equal
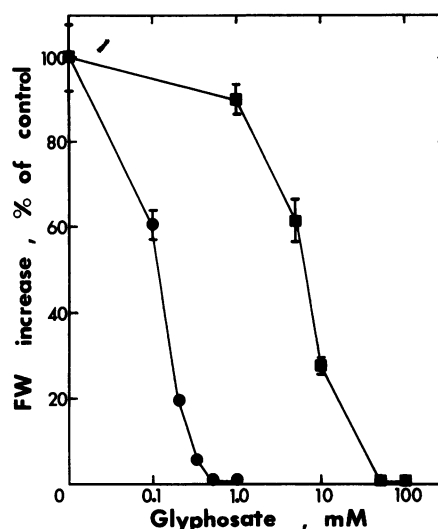

FIG. 1. Effect of glyphosate on the growth of glyphosate-adapted and nonadapted carrot cells, incubated in liquid medium for 10 d. Fresh weight increases for controls (no glyphosate) were 15.5 and 7.7 g/flask for nonadapted and adapted cells, respectively. (●), nonadapted; (■), glyphosate-adapted. Vertical bars are ±SE. FW, fresh weight.
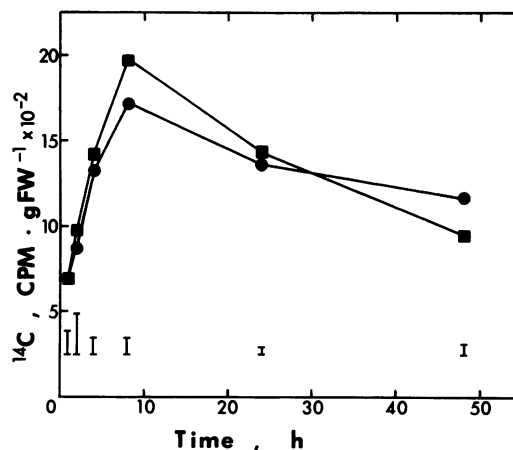

FIG. 2. Uptake of [¹⁴C]glyphosate by glyphosate-adapted and nonadapted carrot cells inoculated into fresh medium. Medium contained 910 cpm/ml and glyphosate concentration was 0.25 mM. (●), nonadapted; (■), glyphosate-adapted. Vertical bars represent difference required for significance at P = 0.05, FW, fresh weight.

to the concentration in the medium. There were no striking differences in the uptake kinetics of the two cell types.

Amino Acids. By 4 d after inoculation, total free amino acid content of adapted cells was 94% higher than in nonadapted cells (Table I). The amino acids threonine, methionine, tyrosine, phenylalanine, tryptophan, histidine, and arginine were present in the adapted cells at levels 4- to 5-fold those in nonadapted cells, while levels of glutamic acid and glutamine in nonadapted cells were about 2-fold those in adapted cells.

In nonadapted cells, glyphosate caused an increase in total free amino acids, to 39% above the untreated control level by 48 h after treatment (Table I). Much of this was due to increased amounts of asparagine, glutamic acid, and glutamine in treated cells. Threonine, cystine, isoleucine, and free ammonium levels were also increased by glyphosate. Amino acids whose levels decreased as a result of glyphosate treatment in nonadapted cells included serine, glycine, methionine, and phenylalanine. Tyrosine, tryptophan, and arginine levels were also decreased by about 50 to 60% but the decrease was not statistically significant due

Table I. *Effect of Glyphosate on Free Amino Acid and Ammonia Levels of Glyphosate-Adapted and Nonadapted Carrot Cells*

The number in parentheses is the standard deviation ($n = 4$ at 0 h, and $n = 2$ at 48 h).

Amino Acid	Nonadapted					Adapted					
	0 h		48 h		48 h + 0.25 mM glyphosate		0 h		48 h		48 h + 0.25 mM glyphosate
					nmol/g fresh wt						
Asp	294	(16)	202	(35)	395	(86)	224	(48)	164	(6)	129	(3)
Thr	356	(31)	340	(52)	504	(45)	1,580	(15)	789	(82)	656	(7)
Ser	396	(47)	334	(38)	145	(4)	791	(28)	318	(29)	290	(11)
Asn	2,270	(281)	4,010	(753)	7,700	(470)	2,420	(76)	1,830	(243)	1,690	(63)
Glu	2,100	(339)	583	(3)	1,230	(226)	1,190	(107)	337	(41)	292	(1)
Gln	3,480	(105)	3,620	(327)	9,170	(333)	1,720	(57)	497	(89)	528	(49)
Pro	39	(9)	21	(3)	25	(3)	71	(7)	5	(7)	5	(7)
Gly	134	(11)	162	(12)	91	(3)	268	(11)	93	(14)	91	(12)
Ala	1,430	(178)	680	(43)	762	(55)	1,410	(110)	192	(13)	331	(36)
Val	646	(59)	1,120	(196)	1,310	(93)	1,210	(14)	920	(81)	806	(41)
Cys	53	(1)	60	(0)	135	(15)	59	(3)	61	(0)	88	(10)
Met	129	(24)	363	(55)	124	(5)	991	(13)	842	(36)	731	(41)
Ile	199	(21)	202	(37)	349	(25)	503	(8)	325	(29)	319	(26)
Leu	130	(18)	178	(37)	184	(16)	335	(13)	256	(16)	220	(9)
Tyr	90	(14)	192	(46)	96	(5)	563	(8)	626	(55)	541	(59)
Phe	109	(13)	194	(46)	89	(14)	459	(9)	610	(60)	510	(6)
Trp	21	(3)	52	(13)	23	(3)	112	(5)	123	(3)	102	(19)
Lys	103	(49)	287	(45)	218	(14)	394	(19)	374	(24)	371	(12)
His	330	(51)	432	(48)	405	(2)	1,180	(34)	1,100	(95)	1,050	(69)
Arg	640	(215)	4,720	(1,470)	1,790	(52)	9,640	(140)	10,740	(530)	10,280	(796)
Total	12,950		17,750		24,740		25,120		20,200		19,040	
NH₄	2,364	(288)	751	(276)	1,810	(276)	1,070	(61)	221	(19)	222	(14)

Table II. *Activities of EPSP Synthase, Phosphatase, Anthranilate Synthyase, and Shikimate Dehydrogenase Extracted from Glyphosate-Adapted and Nonadapted Carrot Cells*

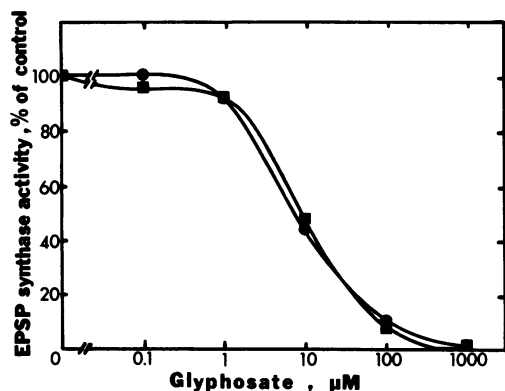| Enzyme	Enzyme Activity		Adapted/ Nonadapted
	Nonadapted	Adapted	
	pkat/mg protein		*ratio*
EPSP synthase	88	1,070	12.2
Phosphatase	530	923	1.7
Anthranilate synthase	12.3	14.8	1.2
Shikimate dehydrogenase	2,417	2,317	1.0

FIG. 3. Effect of glyphosate on EPSP synthase activity from glyphosate-adapted and nonadapted carrot cells. (●), nonadapted; (■), glyphosate-adapted.

to variability among flasks.

Total free amino acid content of adapted cells decreased by about 20% between 4 and 6 d after inoculation, due to decreases in levels of virtually all amino acids except tyrosine, phenylalanine, and tryptophan (Table I). The only statistically significant changes resulting from glyphosate treatment of adapted cells were small increases in the levels of alanine and cystine, and a small decrease in the level of aspartic acid. While not statistically different, the levels of methionine and the aromatic amino acids tended to be lower (by 13–17%) in cells treated with glyphosate. The absolute magnitude of the glyphosate-induced decreases in aromatic amino acids were similar for both cells types: about 90 to 100 nmol/g fresh weight for tryosine and phenylalanine and 20 to 30 nmol/g fresh weight in the case of tryptophan. Decreases in methionine levels resulting from glyphosate treatment were about 240 and 110 nmol/g fresh weight in nonadapted and adapted cells, respectively. Unlike nonadapted cells, adapted cells showed no change in free ammonia level resulting from glyphosate treatment.

Enzymes. EPSP synthase activity in adapted cells was 12-fold that in nonadapted cells, while phosphatase activity was only 70% higher (Table II). Consistent with another report on EPSP synthase from a plant source (13), product formation was linear with time of incubation and with the amount of enzyme used. Glyphosate sensitivities of EPSP synthases extracted from the two tissue types were virtually identical (Fig. 3). In both cases, 50% inhibition occurred with about 10 μM glyphosate.

The activities of anthranilate synthase and shikimate dehydrogenase were quite similar for both cell types (Table II). Anthranilate synthase activity was about 20% higher, and shikimate dehydrogenase about 4% lower in adapted cells compared to nonadapted cells.

DISCUSSION

The adaptation of these cells to glyphosate was similar in several ways to that described by Amrhein *et al.* (3). In both

cases the adaptation was accomplished by transfer into progressively higher glyphosate concentrations, resistance to the herbicide was on the order of 30- to 50-fold, and adapted cells had increased levels of EPSP synthase activity. We have further demonstrated that such adaptations can be stable in the absence of the herbicide. Mammalian cell lines with increased enzyme levels have been found to vary in stability of resistance to the drug methotrexate (1, 10), and stability may provide some clue as to the genetic mechanism involved (1).

Though the increase in EPSP synthase activity of adapted cells occurred in both this and a previously reported case (3), it is impossible to preclude, as was done in the earlier report, the possibility that adaptation resulted from selection for a mutant or epigenetic variant. Though a mutation involving changes in the sensitivity of this enzyme to glyphosate has been reported in a bacterium (6), this is not the mechanism for resistance in this and previous caes (3, 8). However, the stability of the change and the fact that adapted cells grow slowly in the absence of the herbicide prevent any conclusions regarding the genetic nature of this change.

It is clearly demonstrated that this adaptation was not a result of a change in the ability of cells of take up glyphosate from the medium. Since resistance due to reduced uptake of inhibitors has been reported (5, 18), it is important that experiments to test this possibility be included in the initial characterization of resistant lines.

The amino acids present in relatively large amounts in the adapted cells were also those with levels most reduced by glyphosate in nonadapted cells. Also, levels of aromatic amino acids rose between 4 and 6 d in the adapted cells, while the levels of virtually all the remaining amino acids fell or remained constant. These high levels of aromatic amino acids presumably provided protection against glyphosate, in much the same way as did the addition of these amino acids to the medium (9). This is in general support of the hypothesis (2, 4, 6, 15) that inhibition of aromatic amino acid synthesis is the primary mode of action of this herbicide. It remains to be determined why the methionine level was also very high in adapted cells, and was very sensitive to glyphosate in nonadapted cells. This amino acid has not been found effective in reversing glyphosate inhibitions, however (E. D. Nafziger, unpublished data). It could be that adapted cells divert more N through the lysine and threonine/methionine branches of the aspartate pathway, thus reducing the amount of aspartate and N available for asparagine biosynthesis.

While EPSP synthase activity and levels of free aromatic amino acids were both elevated in adapted cells, it is not established that the former caused the latter to occur. Shikimate dehydrogenase activity was not higher in adapted cells, indicating that not all enzymes of the shikimic acid pathway prior to chorismate were similarly affected by the adaptation to glyphosate. Also, anthranilate synthase, which is feedback inhibited by tryptophan (17), had similar activities in the two cell types. Since both the internal concentraton of glyphosate (Fig. 2) and the sensitivity of the enzyme to the herbicide (Fig. 3) were the same in both cell types, the activity of EPSP synthase should have remained substantially higher in the adapted cells. Yet the absolute decrease in aromatic amino acid levels caused by glyphosate was similar for the two cell types. It is clear that an explanation for such effects will be possible only when control of this pathway is better understood. Previous studies have shown feedback control at the branch point chorismate, so increases in enzyme activity before this compound would not be expected to cause great increases in the pathway end products.

Free ammonia levels were quite different, and showed a dif-

ferent response to glyphosate, in the two cell types. The large amounts of arginine and low levels of ammonia in adapted cells suggest that these cells have an effective mechanism to avoid high intracellular ammonia levels. The large increases in glutamine and asparagine and reduction in arginine induced by glyphosate, along with the increase in ammonia, imply that nonadapted cells may have a less effective means of protection against possible ammonia toxicity. While ammonia toxicity has been discussed as a possible consequence of glyphosate treatment (11, 14), this question has not yet been approached in a systematic way.

As another example of high EPSP synthase activity corresponding with tolerance to glyphosate, the present report supplies additional evidence that this enzyme is the target of this herbicide. However, questions regarding the overproduction of aromatic amino acids, and in particular the control of the shikimate pathway, remain unresolved. Additional work is underway to determine the genetic nature of this adaptation.

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